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Antiproliferative Activity of Whey and Casein Bioactive Peptides on Breast Cancer: An In Vitro and In Silico Study

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Abstract

Bioactive peptides from milk proteins are dietary components with various biological properties and therapeutic effects in a variety of health conditions, including cancer. In this study, the effects of goat milk proteins and bioactive peptides on the MCF-7 breast cancer cell line were investigated. Milk was separated into casein and Whey fractions and treated with either pepsin or trypsin enzymes. Peptides of the hydrolyzed fractions were identified with LC-QTOFF/MS. After identification, the hydrolyzed Whey and casein fractions were incubated with MCF-7 breast cancer cells at various concentrations. The in vitro cytotoxicity of hydrolyzed fractions against MCF-7 cells was tested using MTT assay and flow cytometry analysis. The dead MCF-7 cells were analyzed with LC-Q-TOF/MS. In dead MCF-7 cells, the pyruvate kinase M2, mucin 1-C, glycogen synthase kinase 3-β, and L-lactate dehydrogenase B were found to be downregulated in the LC-Q-TOF/MS analysis, while receptor-interacting serine/ threonine-protein kinase 1 was found to be up-regulated. These enzymes and mucin-1C also interacted with the milk-derived bioactive peptides in silico. The goat milk bioactive peptides exhibited high binding affinity with these enzymes and mucin-1C. The 5 μg mL^{-1} trypsin-treated casein fraction caused the highest MCF-7 cell death. In conclusion, the bioactive peptides of the pepsin-treated casein fraction and trypsin-treated Whey fraction of goat milk may inhibit the survival of breast cancer cells and exert their effects via multiple mechanisms. The results of this study are novel, suggesting that goat milk-based specific bioactive peptides may have potential as an anti-tumour agent in the treatment of breast cancer.

Keywords Goat milk · Casein · Whey proteins · Bioactive peptides · Breast cancer

Introduction

Breast cancer is one of the most common cancer types among women. It ranks second in cancer-related deaths after lung cancer (Boyle and Levin 2008). The mechanisms underlying the development of heterogeneous breast cancer, both genetically and histopathologically, remain uncertain (Hedenfalk et al. 2002), and therefore the effect of traditional chemotherapy, and surgery, or radiation for therapeutic purposes is limited. On the other hand, specific natural or synthetic chemical compounds for preservation continue to be widely applied to prevent the cause of cancer formation or to suppress the malignancy of cancer (Sarkar and Yw 2007). It is necessary to develop new chemopreventive agents that can inhibit cancer proliferation and induce apoptosis in cancer cells but have no side effects with inhibition of proapoptotic and antiapoptotic genes for the inhibition of cancer development (Korashy et al. 2012). Current breast cancer treatments like surgery, endocrine therapeutics, radiation therapy, metastasis, and acquired internal endocrine resistance continue to pose some difficulties in the treatment of breast cancer. Accordingly, there is a need for new therapeutic agents with a new mechanism of action and effects on breast cancer (Deng et al. 2018).

This study aimed to investigate the effects of milk-derived peptide fractions on breast cancer cells. It was also aimed to

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support the data obtained from biochemical and cell culture analyses with in silico analyses to evaluate the cell death mechanisms. The interactions of pepsin casein and trypsin Whey fractions with pyruvate kinase isoform (Yang et al.), mucin 1, glycogen synthase kinase 3-β, receptor-interacting serine/threonine-protein kinase 1, L-lactate de-hydrogenase B enzymes of breast cancer cells were determined by in silico docking analysis as these enzymes have been associated with breast cancer.

An isoform of pyruvate kinase, PKM2, is highly expressed in human cancers. The level of this enzyme is higher in cancer cells compared to healthy cells. PKM2 in tumors promotes low private kinase activity, which is required for cancer cell growth and proliferation (Yang et al. 2012; Sciacovelli et al. 2014). The inhibition of PKM2 downregulates glycolysis (Silvestri et al. 2015). The mucin 1 (Kosugi et al.). protein is overexpressed in many human cancers. C terminal of MUC1 (MUC1-C) has a role in glucose uptake regulation and lactate production in human breast cancer cells. Kim et al. have revealed that MUC1-C interacts with multiple signalling receptors, making it an important molecule in the cancerogenic process (Kim et al. 2020). The cytoplasmic domain of MUC1-C interacts with PKM2 and regulates its activity. The MUC1-C activates the PKM2 activity by binding to its cysteine residue (Kosugi et al. 2011). Glycogen synthase kinase-3β (GSK-3β) is the isoform of GSK-3. It modulates glycogen synthesis and regulates protein synthesis, immune function, inflammation, cell differentiation and proliferation, and neuronal signalling (Beurel et al. 2015). GSK-3β also promotes the degradation of several proteins involved in cell proliferation and, therefore, its inhibition induces apoptosis and inhibits the proliferation of MCF-7 cells (Chu et al. 2011). Receptor-interacting serine/threonine-protein kinase 3 (RIPK3) is a molecule involved in the signalling of necroptosis and stimulates anticancer immune responses in mouse models of chemotherapy. RIPK1 is another kinase of the same family and activates RIPK3 (Stoll et al. 2017). The majority of cancer cell lines lack RIPK3 and RIPK1 expression, so the stimulation of their expression can stimulate the necroptosis (Khorsandi et al. 2017) The B chain of the lactate dehydrogenase enzyme is called lactate dehydrogenase B (LDHB), and it catalyses the interconversion of pyruvate and lactate in post-glycolysis. LDHB expression is related to the metabolic characteristics of breast cancers. This property of LDHB may contribute to the selection of new treatment modalities (Dennison et al. 2013). Studies have opened new research areas on bioactive substances from food, and pioneering work in this field has been the basis of enriched food and nutrition science. Food-borne bioactive substances are components that can perform regulatory activities in the human organism beyond basic nutrition (Clare and Swaisgood 2000; FitzGerald and Meisel 2003).

Over the past decade, the interest in milk-based proteins and their bioactive peptides has increased. Milk-based bioactive peptides exhibit immunomodulatory, opioid, antioxidant, and antibacterial activities (Meisel and FitzGerald 2003; Brandelli et al. 2015; Osman et al. 2016). The protein composition of goat milk is similar to breast milk when compared to cow milk (Lara-Villoslada et al. 2004; Prosser 2021). It contains a variety of bioactive peptides with antioxidant, antidiabetic, and antihypertensive effects (Ahmed et al. 2015; Osman et al. 2016). Bioactive peptides are also thought to have some favourable effects the breast cancer treatment (Soudy et al. 2011). Therefore, the effects of goat milk-based bioactive peptides on breast cancer cells were investigated with biochemical and in silico approaches in this study.

Material and Methods

Goat Milk

Milk samples were purchased from Saanen-Maltese crossbreed goats. Goat milk from Saanen- Maltese crossbreed goats were purchased from the farm producing goat milk according to the Turkish Food Codex Communiqué on Drinking Milk with the communiqué numbered 30699 2019/12. Ethical permission is not required for goat milk samples used in this study, according to rule 8 k of the Regulation on Working Procedures and Principles of Animal Experiments Ethical Committees dated 15/02/2014 and numbered 28914.

Goat milk protein, fat, dry matter, and ash levels were determined according to the Association of Analytical Communities (International et al.) (International et al. 2010).

Preparation and Analysis of Goat Milk Protein Hydrolysates

The milk sample was diluted with distilled water in a 1:15 ratio. 1 N NaOH was used to adjust the diluted milk H to 9.5. Thereafter, the milk was kept at room temperature for an hour and was centrifuged for 30 min at ×5000*g*. 1 N HCl was used to adjust the pH of the supernatant (Whey proteins) to 4.5. The supernatant (Whey proteins) and precipitate (casein) were then lyophilized.

Hydrolysis of Goat Milk With Pepsin and Trypsin Enzymes

10 mg mL^{-1} lyophilized goat milk Whey and casein fractions were digested with pepsin (24 h at 25 °C) or trypsin (24 h at 37 °C) in a 20:1 ratio. The enzymatic hydrolysis was

stopped by incubating them at 98 °C for 10 min. (Ababukar et al. 1998). The prepared milk hydrolysates were then stored at -80 °C.

LC–Q–TOF–MS Analysis of Digested Milk Proteins and MCF-7 Cells

Proteins were extracted with an extraction kit (Expedeon-44101, Lucerna-Chem, Switzerland) and digested (Expedeon-44250, Lucerna-Chem, Switzerland) to obtain the peptides. The obtained proteins were identified with the LC–MS system (Waters, Xevo G2-XS, USA). MCF-7 cells were also subjected to LC–MS proteomic profiling using this system.

MCF-7 Cell Culture and Incubation of MCF-7 Cells with the Hydrolyzed Goat Milk Fractions

The MCF-7 breast cancer cell line was purchased from ATCC (American Type Culture Collection). MCF-7 breast cancer cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine in a humidified, 5% CO_2 and 37 °C atmosphere. Cell proliferation was determined by the MTT assay to investigate the effect of hydrolyzed goat milk on the viability of MCF-7 breast cancer cells. 5, 10, 25, 50, and 100 µg mL^{-1} hydrolyzed goat milk was incubated with MCF-7 cell lines. After 24 and 48 h of the incubation, the cellular viability was determined with the MTT assay (Gasparini et al. 2017). The positive control was MCF-7 cells incubated with 10 µg mL^{-1} 5-FU.

Progenesis Analysis of the MCF-7 Cells

After the LC–Q–TOF–MS analysis of MCF-7 cells, the progenesis LC–MS software was used to analyze RAW files. By aligning each LC–MS run to a reference file, this software extracts quantitative information from MS1 data. Statistical analysis was used to filter the results. Any peptides with a $p > 0.05$ ANOVA score were excluded. The remaining peptides' MS2 data was exported, and the resulting MGF file was used to lookup proteins in the Swiss-Prot database (Release 2011_05) on the MASCOT server (http://www.matrixscience.com) for peptide identification. Peptide identifications were entered into the Progenesis software and assigned to features that matched. Peptides with a Mascot score of less than 40 were excluded, and the peptides were assigned to a protein. Proteins containing quantitative information from two or more peptides were accepted as significant.

Flow Cytometric Analysis

The death type of MCF-7 cells after the incubation with hydrolyzed fractions was determined by a flow cytometer (FACS Calibur 4CS, BD, USA). The MCF-7 cells were trypsinized and 2×105 cells were incubated for 24 h at 37 °C. Then the cells were incubated with different concentrations of goat milk-based casein and Whey fractions for 24 h. 400 µL cellular suspension was mixed with 2 µL Annexin-V (1 mg mL^{-1}) and PI (1 mg mL^{-1}). FL1 (Green530/30) and FL2 (Yellow-585/42) filters were used for flow cytometric analysis (Wlodkowic et al. 2009). Annexin-V and PI (−) MCF-7 cells were examined as alive. Annexin-V (+) and PI (−) MCF-7 cells were examined as apoptotic, and Annexin-V (+) and PI (+) MCF-7 cells were examined as of late apoptotic or necrotic (Fadok et al. 1992; Darzynkiewicz et al. 1997).

In Silico Bioactive Peptide Determination

The milk-based peptides were evaluated with the Peptide Ranker program (Mooney et al. 2012). PeptideRanker was used to detect the probable bioactive peptides. Peptides with scores above 0.5 were accepted as bioactive (Imai et al. 2021). The BioPep tool was used to obtain the biological activities of the peptides (Table 2).

Molecular docking of casein and Whey fraction derived peptides and the pyruvate kinase isoform, mucin 1, glycogen synthase kinase 3-β, receptor-interacting serine/ threonine-protein kinase 1, L-lactate dehydrogenase B

The HPEPDOCK molecular docking server (Zhou et al. 2018) was used to dock the obtained goat milk-based bioactive peptides with pyruvate kinase M2 (Protein Data Bank (PDB): 3GR4), mucin 1(PDB: 6BSC), glycogen synthase kinase 3-β (PDB: 1J1B), receptor-interacting serine/ threonine-protein kinase 1 (PDB: 4ITJ), L-lactate dehydrogenase B (PDB: 1T2F). The peptide flexibility is taken into account in the HPEPDOCK docking process by using the MODPEP tool to generate an ensemble of peptide conformations. Then, using MDock's rigid docking procedure, the sampled peptide conformations are globally docked against the entire protein. By producing a large number of peptide conformations, HPEPDOCK is able to consider peptide flexibility. The HPEPDOCK server accepts both sequences and structures as input, and may automatically integrate available peptide-binding information from the Protein Data Bank (PDB).

For the docking study; The protein to be interacted with the peptide was loaded as a pdb file. The peptide sequence is copied and pasted in FASTA format. FASTA-formatted

Table 1 Protein concentration of casein and Whey fractions of goat milk

	Casein fraction		Whey fraction	
	Mean	SD	Mean	SD
Protein (% m/m)	85,91*	2,55	46,94	3,17

SD standard deviation

*$p < 0.05$ compared to Whey fraction, n = 10

peptide sequence file was uploaded. Then the server presented the docking scores of the protein to have interacted with milk-derived peptides. Although there is no maximum length for the peptide, the server suggests 30 amino acid sequences for accuracy.

These molecules were subjected to in silico molecular docking studies because these specific enzymes and proteins were found to be up-or down-regulated based on data obtained from LC–MS analysis of dead MCF-7 cells in this study, and because it was stated in the literature that these proteins and enzymes play a role in MCF-7 cell death.

Statistical Analysis

Data generated in this study were shown and plotted as the sample mean ± its corresponding standard deviation, obtained from at least 10 replicate experiments. For cell culture experiments, at least 6 replicate experiments were carried out. SPSS 20 package statistical analysis program was used for statistical analysis. For the ANOVA test, $p < 0.05$ was accepted as significant. IC50 values were calculated using the AAT Bioquest IC50 calculator (AAT Bioquest, Inc. Quest Graph IC50 Calculator. AAT Bioquest https://www.aatbio.com/tools/ic50-calculator).

Results

Goat Milk Protein, Fat, Ash, Dry Matter, and pH Results

Goat milk protein level was 3.84% w/w, the fat level was 4% w/w, ash was 1.1% w/w dry matter was 12.8% w/w. The pH of the goat milk was 6.7.

Total Protein Levels of Milk Protein Fractions

Protein percentages of casein and Whey fractions obtained from goat milk were presented in Table 1. Accordingly, the protein level of the casein fraction was significantly higher than the protein level of Whey fraction values ($p < 0.05$).

The Bioactive Peptides of the Pepsin and Tyrpsin-Treated Fractions

In this study, the highest MCF-7 cell death was observed in pepsin-treated casein and pepsin-treated Whey fractions. Therefore, further results were presented with these fractions.

The bioactive peptides of the pepsin-treated fractions were DYRWIAL, QIMSSPWGEMYNIF, CDELGIMIWQDF, QYPYQGPIVL, and SSGLGNVPRPYQL. Among these bioactive peptides, DYRWIAL has the highest Peptide Ranker bioactivity score.

The bioactive peptides of trypsin-treated fractions were QYKIPDWFLNR, ALPMHIR, and LVMFQRR. Among these bioactive peptides, QYKIPDWFLNR has the highest Peptide Ranker bioactivity score. In Table 2, probability scores of peptides found to be bioactive were presented.

The general properties of the detected bioactive peptides were determined as an ACE inhibitor, an antioxidative, neuropeptide, and a DPP4 inhibitor.

Anti-proliferative Activity of Pepsin and Tyrpsin-Treated Casein Fractions

When cell viability tests were performed for 24 and 48 h, 24 h incubation of MCF-7 cells with 5 µg mL^{-1} trypsin-treated casein fraction caused the highest cell death (Fig. 1). The IC50 values (the concentration of peotide required to achieve 50% cell viability in MCF-7 cells) were presented at Table 3. The lowest IC50 value was observed in the trypsin-treated casein fraction at 24 h.

Flow cytometric analysis was used to determine the death type of MCF-7 cells incubated with untreated casein, trypsin and pepsin-treated casein. 5, 10, 50 and 100 µg mL^{-1} pepsin-treated casein caused lower necrotic and apoptotic death in MCF-7 cells compared to related trypsin-treated casein fractions. The positive control was MCF-7 cells incubated with 10 µg mL^{-1} 5-FU.

Annexin V (+) and PI (+) cell percentages were the highest in MCF-7 cells incubated with tyrpsin-treated casein fraction compared to MCF-7 cells incubated with 5-FU (Fig. 2). Higher apoptotic MCF-7 cell death was detected with the incubation with the intact casein fraction compared to the pepsin-treated casein fraction. The MCF-7 cell viability percentage decreased when incubated with the tyrpsin-treated casein fraction (Fig. 2).

Anti-proliferative Activity of Pepsin and Tyrpsin-Treated Whey Fractions

When 24 and 48-h cell viability tests were performed, the cell death in MCF-7 cells incubated for 24 h with

Table 2 Sequences of potential bioactive goat milk peptides

Potential bioactive Peptide sequences*	Peptide ranker scores	Potential biological activities (BIOPEP-UWM)+
Pepsin-treated casein fraction		
DYRWIAL	0.75	ACE inhibitor, Antioxidative, neuropeptide, DPP4 inhibitor
QIMSSPWGEMYNIF	0.67	ACE inhibitor, Antioxidative, DPP4 inhibitor
CDELGIMIWQDF	0.62	ACE inhibitor, Antioxidative, DPP4 inhibitor
QYPYQGPIVL	0.59	ACE inhibitor, a DPP4 inhibitor, Antithrombotic, Antioxidative, alpha-glucosidase inhibitor, Antiinflammatory
SSGLGNVPRPYQL	0.55	ACE inhibitor, a DPP4 inhibitor, Antioxidative, Antiinflammatory
Trypsin-treated Whey fraction		
QYKIPDWFLNR	0.82	ACE inhibitor, a DPP4 inhibitor, Renin inhibitor
ALPMHIR	0.63	ACE inhibitor, a DPP4 inhibitor, Renin inhibitor, Antioxidative
LVMFQRR	0.51	ACE inhibitor, a DPP4 inhibitor

*Potential bioactive peptides were evaluated by the Peptide Ranker (p > 0.5)

+ BIOPEP-UWM: https://biochemia.uwm.edu.pl/biopep-uwm/

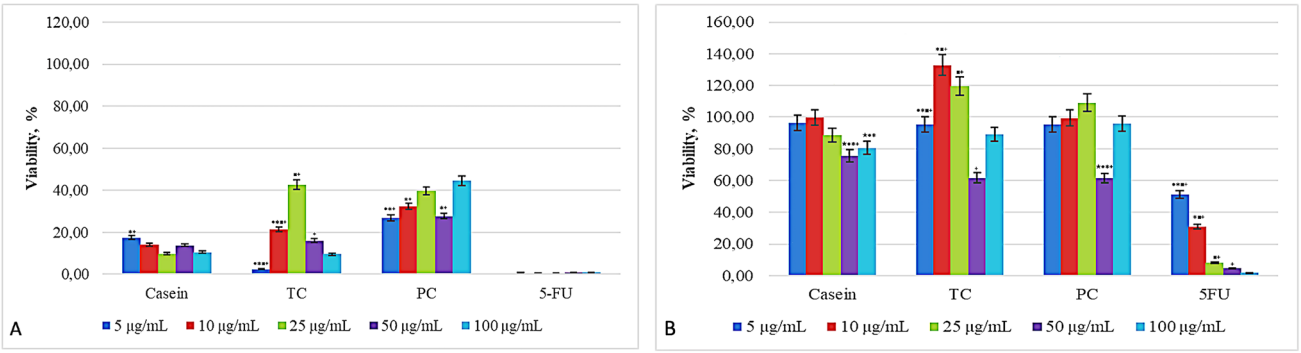


Fig. 1 Viability Percentage (MTT) of MCF-7 breast cancer cells after 24 and 48 h incubation with pepsin and tyrpsin-treated casein fractions. **A** 24 h later than the incubation **B** 48 h later than the incubation. Black Star: $p < 0.05$ compared to 5 µg mL−1, Black circle: $p < 0.05$ compared to 10 µg mL−1. *$p < 0.05$ compared to 25 µg mL−1, Black square: $p < 0.05$ compared to 50 µg mL−1. +$p < 0.05$ compared to 100 µ.g mL−1

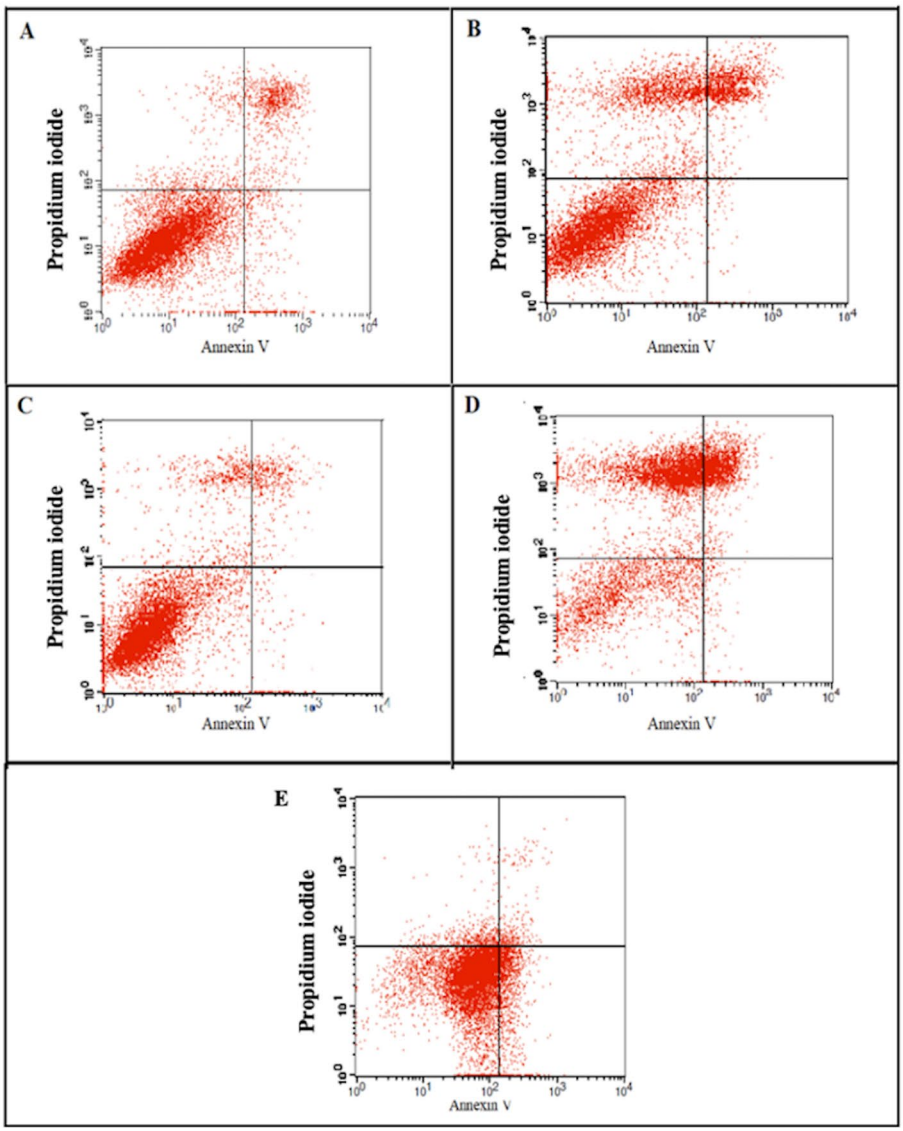
Table 3 IC50 values of the Whey, casein fractions and their hydrolsates

	24 h incubation IC50 values (µg mL^{-1})	48 h incubation IC50 values (µg mL^{-1})
Whey	23.62	4.54
Pepsin-treated Whey	98.30	33.38
Trypsin-treated Whey	32.93	23.49
Casein	10.10	25.19
Pepsin-treated casein	51.86	34.83
Trypsin-treated casein	6.57	34.23

100 µg mL^{-1} untreated Whey and also pepsin-treated Whey fractions was the highest (Fig. 3). Among the Whey fractions, the lowest IC 50 value was detected in intact Whey fraction both at 24 h and 48 h (Table 3).

Incubation of MCF-7 cells with 100 µg mL^{-1} tyrpsin-treated Whey fraction caused higher apoptotic cell death when compared to pepsin-treated Whey fraction.

The Annexin V (+) and PI (+) cell percentages were higher in MCF-7 cells incubated with 100 µg mL^{-1} pepsin-treated Whey compared to the MCF-7 cells incubated with 5-FU (Fig. 4). Untreated Whey fraction caused higher apoptotic cell death when compared to pepsin-treated Whey fraction. The highest necrotic MCF-7 cell death percentage was detected in the MCF-7 cells incubated with 100 µg mL^{-1} tyrpsin-treated Whey fraction compared to other treatments (Fig. 4).



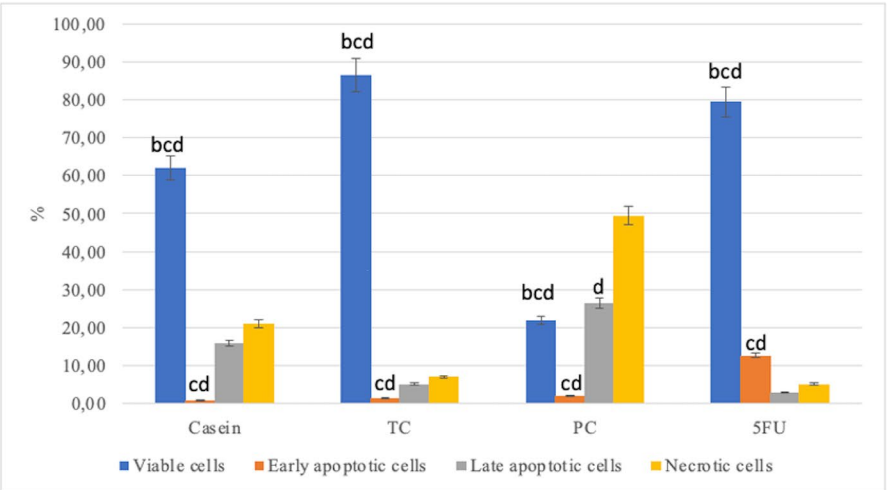


Fig. 2 Analysis of MCF-7 breast cancer cells death type by flow cytometry that was treated with casein proteins and casein peptide fractions. **A** Control Cells, **B** Casein Proteins, **C** Pepsin-treated casein fraction, **D** Tyrpsin-treated casein fraction, **E** 5-Fluorouracil. The X-axis of the diagram shows distributions according to FITC Annexin V radiation, and the Y-axis according to PI radiation. Each red dot represents a cell. Percentages indicate the ratio of the number of cells in the relevant region of the diagram to all of the cells counted (X-axis Annexin V, Y-axis PI signal). Lower left panel (Annexin V−/PI−) shows live cells; lower right panel (Annexin V+/PI−) early apoptotic cells; top right panel (Annexin V+/PI+) late apoptotic cells; upper left panel (Annexin V−/PI+) necrotic cells). *b* $p < 0.05$ compared to early apoptotic cells, *c* $p < 0.05$ compared to late apoptotic cells, *d* $p < 0.05$ compared to necrotic cells

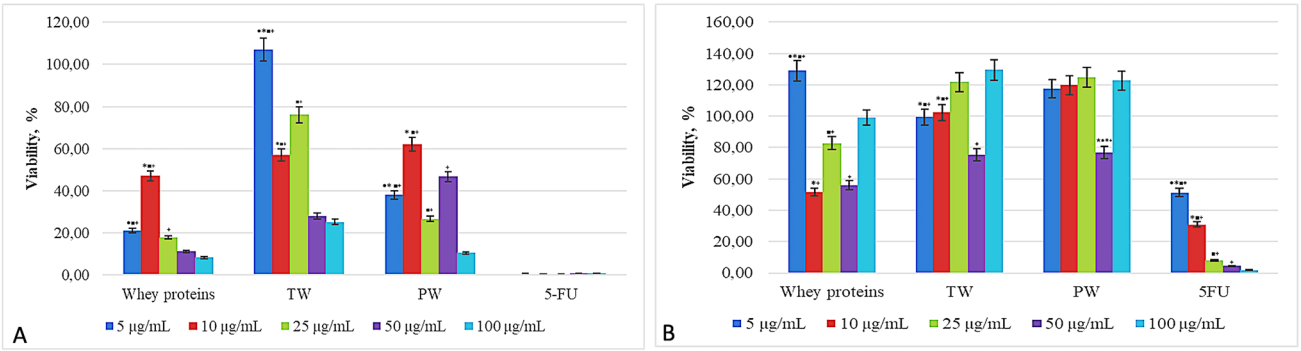


Fig. 3 Viability Percentage (MTT) of MCF-7 breast cancer cells after 24 and 48 h incubation with pepsin and tyrpsin-treated Whey fractions. **A** 24 h later than the incubation **B** 48 h later than the incubation. Black Star: $p < 0.05$ compared to 5 µg mL−1, Black circle: $p < 0.05$ compared to 10 µg mL−1. *$p < 0.05$ compared to 25 µg mL−1, Black square: $p < 0.05$ compared to 50 µg mL−1. +$p < 0.05$ compared to 100 µ.g mL−1

Molecular Docking Studies

Docking analysis with enzymes and peptides that play a role in the death mechanisms of MCF-7 cells allowed us to determine the probable underlying mechanisms of MCF-7 cell death. In the molecular docking analysis of pepsin-treated casein fraction, CDELGIMIWQDF peptide showed the highest docking score with GSK 3-β and MUC-1. DYRWIAL peptide presented the highest docking score with PKM2, QIMSSPWGEMYNIF peptide showed the highest docking score with RIPK-1, and SSGLGNVPRPYQL peptide had the highest docking score with LDH B (Table 4).

The high docking score means the docking of either GSK 3-β, PKM2, RIPK-1, LDH B or MUC-1 with the bioactive peptides of hydrolyzed casein or Whey fractions demonstrated well-situated bonds with the amino acids found in the active site.

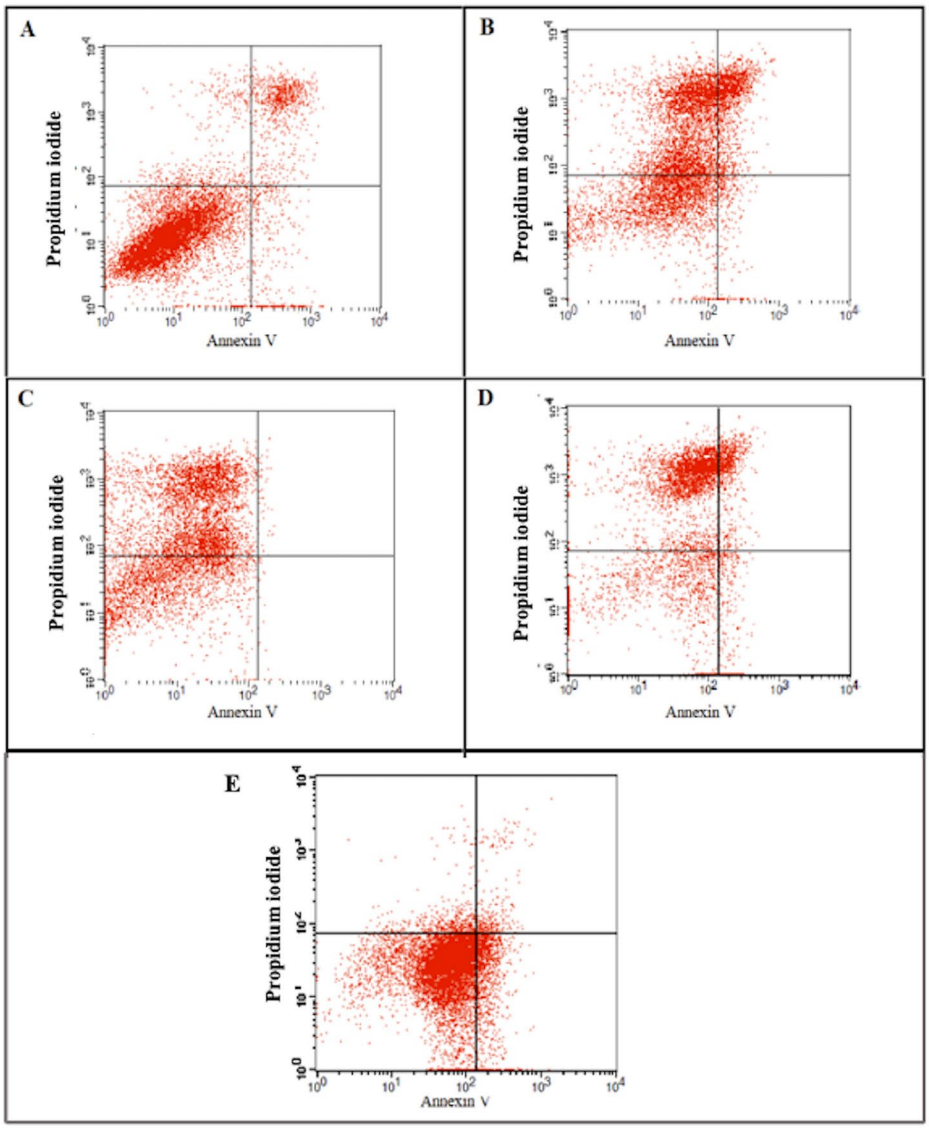
In the molecular docking analysis of bioactive peptides of trypsin-treated Whey fraction, QYKIPDWFLNR peptide interacted with the GSK 3-β, PKM2, RIPK1 and MUC 1, ALPMHIR peptide interacted with the LDH B with the highest docking scores (Table 4). Figure 5 shows the predicted top potent docked structure of the peptides with glycogen synthase kinase 3-β.

Discussion

The basic properties of cancer cells depend on their interactions with their microenvironment. Breast cancer cells interact with surrounding components such as vascular endothelial cells, fibroblasts, immune cells, and mast cells. By inhibiting the components that play a role in this interaction, the survival of cancer cells can be prevented (Allinen et al. 2004). Peptides are ideal candidate inhibitors in cancer therapy to develop an alternative treatment strategy. They can cause cancer cell death without affecting the healthy cells. Peptide-based hormone treatment has been extensively researched and used for breast cancer treatment (Thundimadathil 2012).

In the present study, the effects of goat millk-based casein and Whey fraction and their pepsin and trypsin hydrolysates on the MCF-7 breast cancer cell line have been investigated with biochemical and in silico approaches. This study used complementary in silico analysis to gain a larger view of the individual protein alterations obtained by LC/MS analysis and, as a result, identified novel interactions that could be evaluated as sensible targets. In a study designed similar to this study, luteinizing hormone-releasing hormone (LHRH) agonists, goserelin (XHWSYKLRPG), histrelin (XHWSYXLRP) and triptorelin (XHWSYKLRPG) have been found to have anti-carcinogenic potential in breast cancer cells (Thundimadathil 2012).

In this study, goat milk Whey and casein fractions were hydrolyzed with trypsin and pepsin separately to obtain the bioactive peptides. Enzymatic hydrolysis was used for the bioactive peptide extraction as the reaction time is shorter and non-toxic intermediate products are generated (Lin et al. 2012). Bioactive peptides may present anticancer properties (Chiangjong et al. 2020) as their amino acids may have the potential to increase cell permeability (Perry et al. 2018). Pepsin cleaves the peptide bonds of polypeptide chains between the aromatic and hydrophobic amino acids. Positively charged and hydrophobic lysine and arginine-rich peptide chains act as cationic peptide chains that can react with the anionic membranes on cancer cells, can disrupt the integrity of the cell membrane, can penetrate the membrane, and can serve a toxic role on cancer cells. Glycine, lysine, and leucine are the most common amino acid residues in peptides with anticancer properties (Shoombuatong et al. 2018). For example, hydrophobic positively charged lysine- and arginine-rich peptides can interact with membranes via a snorkelling mechanism, choosing anionic membranes



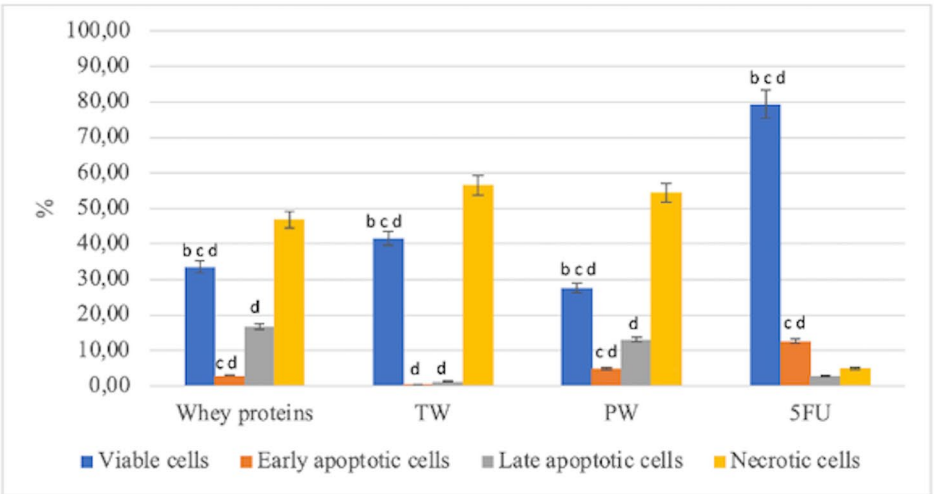


Fig. 4 Flow cytometric analysis of MCF-7 breast cancer cell death type treated with Whey proteins and Whey peptide fractions. **A** Control Cells, **B** Whey Proteins, **C** Pepsin-treated Whey fraction, **D** Tyrpsin-treated Whey fraction, **E** 5-Fluorouracil. The X-axis of the diagram shows distributions according to FITC Annexin V radiation, and the Y-axis according to PI radiation. Each red dot represents a cell. Percentages indicate the ratio of the number of cells in the relevant region of the diagram to all of the cells counted (X-axis Annexin V, Y-axis PI signal). Lower left panel (Annexin V−/PI−) shows live cells; lower right panel (Annexin V+/PI−) early apoptotic cells; top right panel (Annexin V+/PI+) late apoptotic cells; upper left panel (Annexin V−/PI+) necrotic cells). *b* $p < 0.05$ compared to early apoptotic cells, *c* $p < 0.05$ compared to late apoptotic cells, *d* $p < 0.05$ compared to necrotic cells

Table 4 Molecular docking scores of goat milk-based bioactive peptides with GSK 3-β, PKM2, RIPK-1, LDH B enzymes and MUC 1

Peptides	GSK-3 β	PKM2	RIPK-1	MUC-1	LDH B
Pepsin-treated Casein fraction					
CDELGIMIWQDF	− 223.106	− 192.511	− 167.734	− 180.193	− 185.492
DYRWIAL	− 196.354	− 199.784	− 165.229	− 176.588	− 193.059
QIMSSPWGEMYNIF	− 219.710	− 193.818	− 175.743	− 178.251	− 183.476
QYPYQGPIVL	−198.132	− 189.068	− 167.180	− 176.579	− 182.026
SSGLGNVPRPYQL	− 202.117	− 180.711	− 175.390	− 166.259	− 219.055
Tyrpsin-treated Whey fraction					
QYKIPDWFLNR	− 235.416	− 222.176	− 179.917	− 180.122	− 164.732
LVMFQRR	− 196.047	− 174.486	− 179.189	− 164.011	− 185.727
ALPMHIR	− 175.421	− 174.818	− 161.394	− 145.320	− 206.751

GSK-3 β Glycogen synthase kinase-3 beta (PDB: IJ1B), *PKM2* Pyruvate Kinase M2 (PDB:3GR4), *RIPK-1* receptor-interacting serine/threonine-protein kinase 1, *(PDB:4ITJ) MUC-1* Mucin 1, *(PDB: 6BSC) LDH B* L-lactate dehydrogenase B, *(PDB: 1T2F) PDB* Protein Data Bank

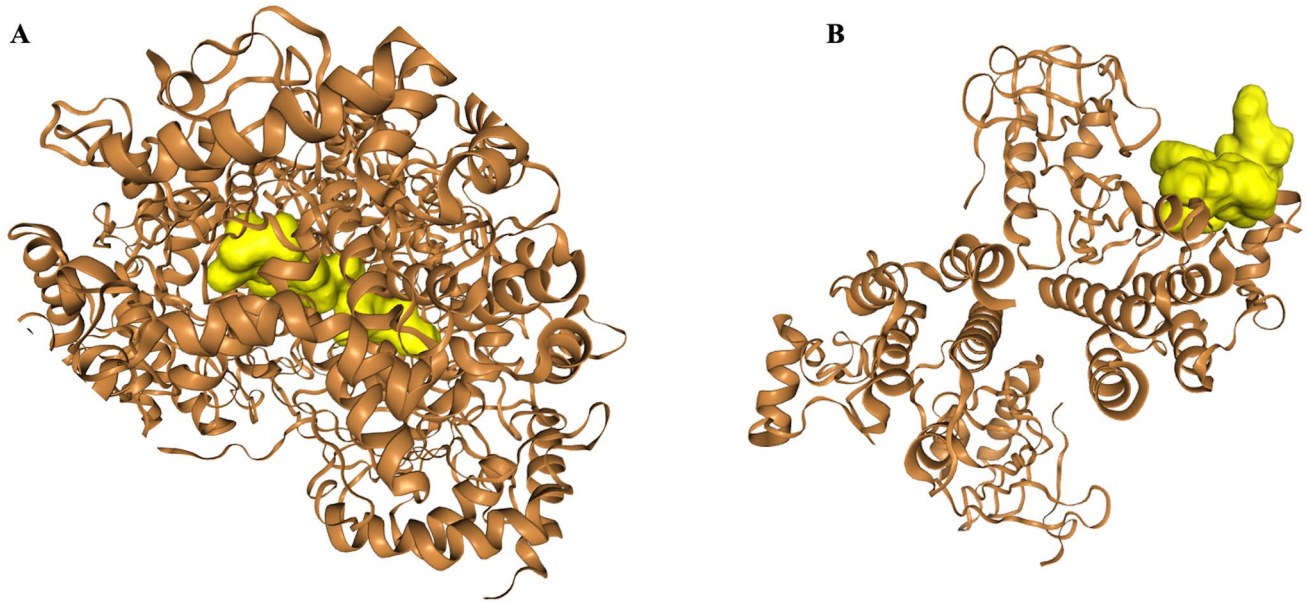


Fig. 5 Predicted top potent docked structure of the peptides with glycogen synthase kinase 3-β. **A** Docking of "CDELGIMIWQDF" peptide from pepsin-treated casein fraction with glycogen synthase kinase 3-β. **B** Docking of "QYKIPDWFLNR" peptide from trypsin-treated Whey fraction with glycogen synthase kinase 3-β

on cancer cells, altering cell membrane integrity, penetrating the membrane, and potentially serving a role in cancer cell toxicity (Dai et al. 2017). Peptides containing phenylalanine can increase their affinity for targeting cancer cell membranes (Dennison et al. 2007; Dai et al. 2017). Furthermore, because histidine is protonated under acidic pH circumstances, histidine-containing peptides can cause cancer cytotoxicity by increasing membrane permeability. In cancer cells, glutamic and aspartic acid residues may have antiproliferative properties (Yamaguchi et al. 2016). Prolines in the peptide structure are important for membrane interaction and conformational flexibility in cancer cells. It is reported that membrane interactions and conformational flexibility increase cytotoxic activity (Liu et al. 2016). In this study, among the peptides obtained from the pepsin-treated casein fraction, the DYRWIAL peptide had the highest bioactivity score. The bioactive peptides of pepsin-treated casein fraction were DYRWIAL, QIMSSPWGEMYNIF, CDELGIMIWQDF, QYPYQGPIVL, and SSGLGNVPRPYQL, respectively, starting with the highest bioactivity score.

The cleavage site of trypsin is the carboxyl side of the lysine or arginine amino acids, except that one of the two is followed by a proline (Keil 2012). The bioactive peptides of trypsin-treated Whey fractions were QYKIPDWFLNR,

ALPMHIR, and LVMFQRR, respectively, starting with the highest bioactivity score.

In the present study, it was found that the bioactive peptides of either pepsin-treated casein fraction or trypsin-treated Whey fraction contain at least one arginine, lysine, or phenylalanine residue. The general properties of the detected bioactive peptides were determined as an ACE inhibitor, an antioxidative and a DPP4 inhibitor in the BIOPEP-UWM. There were no reported results related to the anticancer effects of these bioactive peptides.

Among the problems encountered in the development of therapeutic bioactive peptides, the most common problem is establishing a cause-effect relationship between the consumption of bioactive peptides and the intended health effects in humans. The use of peptides in cancer therapy is associated with different mechanisms that inhibit tumour growth (Thundimadathil 2012). One of the ways used to elucidate these different mechanisms is in silico analysis. Integrating various data sets and algorithms is required to improve our understanding of cancer. Combining in vivo and in vitro data, and in silico models, is critical for overcoming the basic challenges posed by data complexity. In silico analysis also aids in the discovery of underlying molecular pathways (Jean-Quartier et al. 2018). Therefore, in the present study, the possible anticarcinogenic activities of the goat milk-based bioactive peptides were also determined by molecular docking analysis. In this study, although the trypsin-treated casein fraction and pepsin-treated Whey fraction caused the highest MCF-7 cell death within 24 h, the apoptotic cell death rate was low and the necrotic death rate was high in these fractions. Since the fractions with the highest apoptotic death rates were detected in pepsin-treated casein and trypsin-treated Whey fractions, possible approaches were made by examining the interactions of bioactive peptides of these fractions with targeted proteins in silico.

The goat milk-based bioactive peptides were docked with glycogen synthase kinase 3-β (GSK 3-β), pyruvate kinase M2 (PKM2), receptor-interacting serine/ threonine-protein kinase 1 (RIPK-1), L-lactate dehydrogenase B (LDH B) enzymes, and mucin 1 (MUC 1). These molecules were used as a marker as their regulations were changed in the MCF 7 cells that died after being treated with the goat's milk-based bioactive peptides.

In the present study, the GSK 3-β, PKM2, LDH B enzymes, and MUC 1 protein were down-regulated, and the RIPK1 enzyme was upregulated in the dead MCF-7 cells after the incubation with pepsin casein and trypsin Whey fractions. GSK-3 is one of the docked enzymes that is involved in the Wnt and Hedgehog signaling pathway, insulin pathway, neuronal development, transcription, cell division, and death. GSK-3α and GSK-3β are the isoforms of GSK-3 in mammals and catalyze similar catalytic reactions (Beurel et al. 2015). In the present study, it was determined that the GSK-3β enzyme was down-regulated in the dead MCF-7 cells that were incubated with pepsin-treated casein and tyrpsin-treated Whey fractions. The CDELGIMIWQDF peptide and QYKIPDWFLNR peptide exhibited the highest docking scores when they were docked with GSK-3β (PDB: 1J1B) compared to other peptides of goat milk fractions.

Pyruvate kinase activity frequently increases in benign and malignant tissue proliferations. PKM2 exists in fetal and mature tissues except for skeletal muscles and erythrocytes (Yilmaz et al. 2003). After the molecular docking of pepsin-treated casein fraction and tyrpsin-treated Whey fraction with PKM2, DYRWIAL peptide from pepsin casein fraction and QYPYQGPIVL peptide from trypsin Whey fraction were found to have the highest docking scores compared to the other bioactive peptide fractions.

Another enzyme that was in silico analyzed was RIPK1. Activation of RIPK1 promotes cell death in radioresistant breast cancer (Newell et al. 2019; Perez-Añorve et al. 2019). QIMSSPWGEMYNIF peptide from pepsin-treated casein fraction and QYKIPDWFLNR peptide from tyrpsin-treated Whey fraction have interacted with RIPK1 with high docking scores. The docking score of QYKIPDWFLNR was higher than that of QIMSSPWGEMYNIF.

The LDH B enzyme activity of the MCF-7 cells was found to be down-regulated after incubation with pepsin-treated casein and trypsin-treated Whey fraction. LDH B is overexpressed in breast and lung cancer, and it has been considered a potential target to treat these types of cancer (Al-Salam et al. 2021). The docking scores of the SSGLGNVPRPYQL peptide from the pepsin-treated casein fraction and the ALPMHIR peptide from the trypsin-treated Whey fraction were higher than the other peptides. The docking scores of the SSGLGNVPPYQL peptide were higher than those of the ALPMHIR peptide. MUC1 is a heterodimeric protein that is expressed in over 90% of breast tumors (Kufe 2009). In breast cancer cells, MUC1 promotes vascular endothelial growth factor-mediated angiogenesis (Woo et al. 2012). These high docking scores indicate that the hydrolyzed casein or Whey fractions interact with the GSK 3-β, PKM2, RIPK-1, LDH B or MUC-1 through their binding to the active site of these proteins.

In this study, 5 μg mL^{-1} pepsin-treated casein fraction caused significant MCF-7 cell death within 24 h, but late apoptotic death of MCF-7 cells was observed in tyrpsin-treated casein fraction. The Annexin V and PI-positive cell percentages were higher than the positive control in the trypsin-treated casein fraction. Untreated casein fraction also caused higher apoptotic cell death when compared to pepsin-treated casein fraction. This finding indicates the possible existence of bioactive peptides in the non-hydrolyzed Whey fraction.

In conclusion, the bioactive peptides of pepsin-treated casein fraction and tyrpsin-treated Whey fraction of goat milk may inhibit the survival of breast cancer cells and exert their effects via.

multiple mechanisms. The results of this study are novel, suggesting that goat milk-based specific bioactive peptides may have potential as an anti-tumour agent in the treatment of breast cancer.

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Declarations

Conflict of ınterest The authors declare that there is no conflict of interest.

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